

Effects of Genetic Polymorphisms in Metabolic Enzymes on the Relationships between 8-hydroxydeoxyguanosine Levels in Human Leukocytes and Urinary 1-hydroxypyrene and 2-naphthol Concentrations

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Abstract: Effects of Genetic Polymorphisms in Metabolic Enzymes on the Relationships between 8-hydroxydeoxyguanosine Levels in Human Leukocytes and Urinary 1-hydroxypyrene and 2-naphthol Concentrations: Yong-Dae KIM, *et al.* Department of Preventive Medicine, College of Medicine, Chungbuk National University, South Korea—This study was designed to investigate the relationship between environmental exposure to polycyclic aromatic hydrocarbons (PAHs) and oxidative stress, and to evaluate the effects of cigarette smoking and the genetic polymorphisms of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6* on the relationship. The subjects of this study were 105 healthy Korean males without occupational exposure to PAHs. The 8-hydroxydeoxyguanosine (8-OHdG) level in leukocytes, and urinary 1-hydroxypyrene (1-OHP) and 2-naphthol concentrations, were measured by high-performance liquid chromatography. Genetic polymorphisms of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6* were identified by PCR and PCR-RFLP methods. The 8-OHdG level showed a significant correlation with the 1-OHP concentration in all subjects ($p < .001$) and in smokers ($p < .01$), and with the 2-naphthol level in non-smokers ($p < .01$). The 8-OHdG level was significantly higher in smoking rapid acetylators than in smoking slow or intermediate acetylators, and in individuals with the *UGT1A6* wild-type than in those with the *UGT1A6* mutant genotype. Significant positive correlations between 8-OHdG and 1-OHP concentrations were found in subjects with every genotype of the *CYP1A1* and *CYP2E1* genes, with the *GSTM1* null-type, with the *NAT2* genotype of a rapid acetylator, and with the *UGT1A6* wild-type, respectively. The urinary 2-

naphthol level significantly correlated with the 8-OHdG level only in subjects with the *GSTM1* null-type. In conclusion, there is a significant correlation between the 8-OHdG level in leukocytes and the urinary 1-OHP concentration in the population not occupationally exposed to PAHs. This relationship is affected by genetic polymorphisms in PAH metabolic enzymes. (*J Occup Health 2003; 45: 160–167*)

Key words: 8-hydroxydeoxyguanosine, 1-hydroxypyrene, 2-naphthol, Metabolic enzyme, Polymorphism

Reactive-oxygen species (ROS) are produced by environmental agents such as radiation and chemical carcinogens, as well as by endogenous oxygen metabolism^{1–3}. ROS can cause extensive DNA damage, including single-strand breaks and the formation of modified bases such as 8-hydroxydeoxyguanosine (8-OHdG)⁴. The level of 8-OHdG has been used as a marker for the evaluation of DNA damage caused by oxidative stress⁵.

Polycyclic aromatic hydrocarbons (PAHs), which are formed during incomplete combustion of fossil fuels, are widely distributed in our environment⁶. Human exposure to PAHs may occur through smoking, polluted air, food consumption and occupational contact^{7–9}. Urinary 1-hydroxypyrene (1-OHP) and 2-naphthol concentrations are used as biomarkers for exposure to PAHs^{10–12}. The latter has been reported to be a good marker for non-occupational inhalation exposure to PAHs¹².

Previous reports have demonstrated that neonatal urinary 8-OHdG concentrations are significantly associated with maternal exposure to environmental tobacco smoke¹³, and that 8-OHdG levels are high in the lung tissue of individuals exposed to diesel exhaust particles¹⁴. This led to speculation that PAHs could induce ROS formation in the human body. If this were

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the case, the 8-OHdG level would show a positive correlation with urinary concentrations of PAH metabolites. This relationship would be influenced by the genetic polymorphisms of *CYP1A1*¹⁵, *CYP2E1*¹⁶, *GSTM1*^{16, 17}, *NAT2*¹⁷ and *UGT1A6*¹⁸, which have been reported to be involved in the metabolism of PAHs.

In this study, we evaluated the relationship between environmental PAH exposure and oxidative stress, the effects of a smoking habit and the genetic polymorphisms of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6* on the relationship.

Materials and Methods

Study subjects

This study was conducted in accordance with the recommendations outlined in the Declaration of Helsinki. One hundred and five healthy Korean males were enrolled in this study. After having given their written informed consent, they were asked to fill out a standardized questionnaire about demographic factors and their smoking habit. Whole blood and spot urine specimens were collected from each subject and kept at -20°C until analyzed.

Analysis of urinary 1-hydroxypyrene and 2-naphthol

For the analysis of urinary 1-OHP and 2-naphthol concentrations, we used the methods developed by Jongeneelen *et al.*¹⁰ and by Kim *et al.*¹¹, respectively. In short, urine samples were buffered with sodium acetate buffer (pH 5.0), and hydrolyzed enzymatically by using β -glucuronidase with sulfatase activity (Sigma, St. Louis, MO) for 16 h at 37°C in a shaking water bath. After hydrolysis, acetonitrile was added and the samples were centrifuged at 10,000 g for 10 min. A high-performance liquid chromatography (HPLC) system, consisting of a pump (Waters 600E, Millipore, Milford, MA), variable-fluorescence detector (RF-10AxL, Shimadzu, Kyoto, Japan), an automatic injector (L-7200, Hitachi, Tokyo, Japan), and an integrator (Chromatopac C-R3A, Shimadzu, Kyoto, Japan), was used. A 150 mm long reverse-phase column (TSK gel ODS-80TM, Tosoh, Tokyo, Japan) was used for 1-OHP analysis, and a 250 mm long reverse-phase column (J'sphere ODS-H80, YMC, Wilmington, NC) for 2-naphthol analysis. The mobile phase was 58% (v/v) acetonitrile for 1-OHP, and 38% (v/v) acetonitrile for 2-naphthol. Excitation/emission wavelengths used in the detection of 1-OHP and 2-naphthol were 242/388 nm and 227/355 nm, respectively.

DNA isolation and 8-hydroxydeoxyguanosine measurement

An assay of the 8-OHdG level in leukocytes was performed by a method described by Kasai *et al.*¹⁹ with a minor modification. Briefly, DNA was extracted from peripheral blood with a DNA Extractor WB kit (Wako,

Osaka, Japan), digested with nuclease P1 (Sigma, St Louis, MO) and treated with alkaline phosphatase (Sigma, St Louis, MO) at 37°C for 1 h, and injected, after centrifugation, into the HPLC system. All these procedures were performed under dark conditions. The nucleosides were separated on a 0.46×25 cm reverse-phase column (Shodex C18-10B, Shoko, Japan) with 10 mM phosphate buffer containing 10% (v/v) methanol. The 8-OHdG level was determined with an electrochemical detector (Coulchem II Model 5200A, ESA, Chelmsford, MA) and deoxyguanosine (dG) was determined with a UV detector (Model 115, Gilson, France). The level of 8-OHdG was expressed as the number of 8-OHdG per 10^5 dG.

Determination of genotypes

The A4889G polymorphism in exon 7 of *CYP1A1* that results in an Ile \rightarrow Val amino acid replacement at residue 462 was determined by the PCR-RFLP method described by Oyama *et al.*²⁰ Genotyping of the *Rsa* I polymorphism in the 5'-flanking region of *CYP2E1* was carried out according to the method described by Kawamoto *et al.*²¹ with a minor modification. The *GSTM1* polymorphism was determined by the PCR procedure described by Comstock *et al.*²² The genotype of *NAT2* was identified by the method described by Deguchi²³, and the phenotype was classified as either a rapid, intermediate or slow acetylator according to the genotype.

The *UGT1A6* genotyping was performed as described by Ciotti *et al.*²⁴ A sense primer [5'-CCCTCGAGGGGAAATACCTAGGAGCCCTGTGA-3'] and an anti-sense primer [5'-AGGAGCCAAATGAGTGAGGGGAG-3'] were used to amplify exon 1 of the *UGT1A6* gene. The amplified product was digested with endonucleases *Bsm*I and *Fnu*4HI to identify the A to G transition at codon 181 (T181A) and the A to C transversion at codon 184 (R184S) mutations. We classified the *UGT1A6* genotype without any allele containing either T181A or R184S as the wild-type sequence, with other genotypes as the mutant forms.

Statistical analysis

The Student's t-test was used to compare the mean 8-OHdG levels of smokers and non-smokers, and of the different genotypes of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6*, respectively. Pearson correlation coefficients of the 8-OHdG level with age, duration and amount of cigarette smoking, and urinary 1-OHP and 2-naphthol concentrations, were estimated. Correlations between the 8-OHdG level and urinary concentrations of the PAH metabolites were statistically tested with respect to smoking status and the genotypes of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6* genes, respectively. Finally, multiple regression analyses including the smoking status,

the genetic polymorphisms of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6*, and the urinary concentration of 1-OHP or 2-naphthol as the independent variables were performed on 8-OHdG levels in leukocytes.

Results

The distributions of age, 8-OHdG levels and urinary 1-OHP and 2-naphthol concentrations are shown in Table 1. When comparisons were made with respect to the smoking status, urinary 1-OHP and 2-naphthol concentrations were significantly higher in smokers than in non-smokers, but the 8-OHdG level was not.

The level of 8-OHdG in leukocytes significantly correlated with the urinary 1-OHP concentration in all subjects and in smokers, and with the urinary 2-naphthol concentration in non-smokers (Table 2).

Table 3 shows the distribution of the 8-OHdG level in smokers and in non-smokers according to the genetic polymorphisms of the five metabolic enzymes. The level of 8-OHdG was significantly higher in individuals with the *UGT1A6* wild-type than those with the *UGT1A6* mutant-type. Smoking rapid acetylators showed a higher 8-OHdG mean level than smoking slow or intermediate acetylators, but there is no enzyme that showed a significant difference in the 8-OHdG level according to

the genetic polymorphism.

We tested the correlations between the 8-OHdG level, and urinary 1-OHP and 2-naphthol concentrations according to the genetic polymorphism of the five genes. We found significant correlations between the 8-OHdG level and urinary 1-OHP concentration in subjects with the *GSTM1* null-type, the *NAT2* rapid acetylator type and the *UGT1A6* wild-type, respectively. A significant correlation between the 8-OHdG level and urinary 1-OHP concentration was found in every genotype of *CYP1A1* and *CYP2E1*. On the other hand, the correlation between the 8-OHdG level and urinary 2-naphthol concentration was significant only in subjects with the *GSTM1* null-type (Table 4).

In a multiple linear regression analysis including the smoking status and the genetic polymorphisms of *CYP1A1*, *CYP2E1*, *GSTM1*, *NAT2* and *UGT1A6*, and the urinary 1-OHP concentration as the independent variables, only the urinary 1-OHP concentration showed a significant association with the 8-OHdG level (Table 5), but there was no variable that showed a significant association with the 8-OHdG level in the multiple regression analysis that included the urinary 2-naphthol concentration as an independent variable instead of the urinary 1-OHP concentration (Table 6).

Table 1. Geometric means and geometric standard deviations of age, urinary concentrations of 1-OHP and 2-naphthol, and the 8-OHdG level in leukocytes according to smoking status

	N (%)	Age	1-OHP ($\mu\text{mol/mol creat.}^*$)	2-Naphthol ($\mu\text{mol/mol creat.}$)	8-OHdG (/10 ⁵ dG)
Total subjects	105 (100)	25.75 \pm 5.38	0.03 \pm 2.14	1.95 \pm 2.46	2.77 \pm 1.38
Smokers	56 (53)	25.84 \pm 4.65	0.05 \pm 2.14 [†]	3.53 \pm 1.92 [†]	2.86 \pm 1.39
Non-smokers	49 (47)	25.65 \pm 6.16	0.02 \pm 1.65	1.00 \pm 1.90	2.72 \pm 1.36

*: Creatinine, †: p-value < .01 when compared with non-smokers

Table 2. Correlation coefficients for urinary 1-OHP or 2-naphthol concentrations and the 8-OHdG level in leukocytes

Variables	Correlation coefficient	p-value
Urinary 1-OHP concentration ($\mu\text{mol/mol creatinine}$)		
Total subjects (N=105)	0.32	< .001
Smokers (N=56)	0.37	< .01
Non-smokers (N=49)	0.23	NS*
Urinary 2-naphthol concentration ($\mu\text{mol/mol creatinine}$)		
Total subjects (N=105)	0.09	NS
Smokers (N=56)	-0.07	NS
Non-smokers (N=49)	0.40	< .01

*: Not significant

Table 3. Geometric means and geometric standard deviations of the 8-OHdG level in leukocytes according to the smoking status and to genetic polymorphisms in Korean males

Genes	Geometric mean \pm geometric standard deviation (N)		
	Total subjects	Smokers	Non-smokers
CYP1A1			
Ile/Ile	2.86 \pm 1.40 (69)	2.94 \pm 1.43 (34)	2.77 \pm 1.36 (35)
Ile/Val + Val/Val	2.64 \pm 1.31 (36)	2.72 \pm 1.30 (22)	2.51 \pm 1.31 (14)
CYP2E1			
c1/c1	2.92 \pm 1.39 (67)	2.97 \pm 1.36 (41)	2.80 \pm 1.42 (26)
c1/c2 + c2/c2	2.61 \pm 1.32 (38)	2.64 \pm 1.40 (15)	2.59 \pm 1.27 (23)
GSTM1			
Null	2.80 \pm 1.40 (61)	2.94 \pm 1.40 (31)	2.64 \pm 1.40 (30)
Positive	2.75 \pm 1.32 (44)	2.72 \pm 1.36 (25)	2.77 \pm 1.27 (19)
NAT2			
Rapid	2.89 \pm 1.42 (52)	3.00 \pm 1.45 (33)*	2.69 \pm 1.35 (19)
Slow + intermediate	2.69 \pm 1.32 (53)	2.64 \pm 1.27 (23)	2.72 \pm 1.36 (30)
UGT1A6			
Wild-type	2.94 \pm 1.36 (67)*	2.97 \pm 1.35 (39)	2.86 \pm 1.39 (28)
Mutant type	2.53 \pm 1.36 (38)	2.56 \pm 1.43 (17)	2.51 \pm 1.28 (21)

*: p-value < .05 when compared with the other genotype of the same gene

Table 4. Correlation coefficients for the 8-OHdG level in leukocytes and urinary 1-OHP or 2-naphthol concentrations according to the genetic polymorphisms

Genes	N	1-OHP		2-naphthol	
		R	p-value	R	p-value
CYP1A1					
Ile/Ile	69	0.30	< .05	0.10	NS*
Ile/Val + Val/Val	36	0.39	< .05	0.09	NS
CYP2E1					
c1/c1	67	0.29	< .05	0.10	NS
c1/c2 + c2/c2	38	0.42	< .01	0.07	NS
GSTM1					
Null	61	0.44	< .001	0.26	< .05
Positive	44	0.06	NS	-0.17	NS
NAT2					
Rapid	52	0.39	< .01	0.22	NS
Slow + intermediate	53	0.21	NS	-0.04	NS
UGT1A6					
Wild-type	67	0.33	< .01	0.07	NS
Mutant type	38	0.27	NS	0.05	NS

*: Not significant

Table 5. A general linear model of the 8-OHdG level including the smoking status, the five genetic polymorphisms and the urinary 1-OHP concentration as the independent variables

Variables	β^*	S.E.(β) [†]	T [‡]	p-value
Smoking status	0.0699	0.0729	0.959	0.3399
CYP1A1	-0.0402	0.0639	-0.629	0.5309
CYP2E1	-0.0595	0.0639	-0.932	0.3539
GSTM1	-0.0223	0.0604	-0.369	0.7130
NAT2	-0.0697	0.0608	-1.147	0.2544
UGT1A6	-0.0939	0.0642	-1.463	0.1468
Urinary 1-OHP concentration ($\mu\text{mol/mol creatinine}$)	0.1364	0.0467	2.920	0.0044

*: Regression coefficient †: Standard error of regression coefficient ‡: $\beta/\text{S.E.}(\beta)$

Table 6. A general linear model of the 8-OHdG level including the smoking status, the five genetic polymorphisms and the urinary 2-naphthol concentration as the independent variables

Variables	β^*	S.E.(β) [†]	T [‡]	p-value
Smoking status	0.0366	0.0907	0.404	0.6871
CYP1A1	-0.7556	0.0653	-1.158	0.2499
CYP2E1	-0.0826	0.0665	-1.243	0.2170
GSTM1	-0.0267	0.0623	-0.428	0.6697
NAT2	-0.0843	0.0627	-1.344	0.1822
UGT1A6	-0.0996	0.0669	-1.488	0.1399
Urinary 2-naphthol concentration ($\mu\text{mol/mol creatinine}$)	0.0517	0.0485	1.066	0.2889

*: Regression coefficient †: Standard error of regression coefficient ‡: $\beta/\text{S.E.}(\beta)$

Discussion

The level of 8-OHdG in leukocytes is significantly associated with cigarette smoking²⁵⁾, and maternal environmental tobacco smoking increases the urinary 8-OHdG level in neonates¹³⁾. In the present study, the 8-OHdG level was not significantly correlated with variables related to cigarette smoking when considering all subjects, but correlated with the urinary 1-OHP concentration in smokers and with the total duration of smoking in rapid acetylators (data not shown). These facts suggest that PAHs in cigarette smoke could increase the 8-OHdG level in smokers, but it seems to be dependent on genetic polymorphisms in the metabolic enzymes.

The mechanism of ROS generation by PAHs is not fully understood yet, but Cu(I) plays an important role. The hydroxyl radical generated by a Cu(I)-catalyzed Fenton reaction is the primary species responsible for DNA fragmentation by *o*-quinones²⁶⁾. Ohnishi and Kawanishi²⁷⁾ have reported that the reduction by NADH

and auto-oxidation of benzo[a]pyrene-7,8-dione occurs concomitantly with the generation of superoxide anion, which is dismutated to hydrogen peroxide, and hydrogen peroxide in turn interacts with Cu(I) to form a DNA-copper-hydroperoxo complex, which causes DNA damage.

CYP1A1 and CYP2E1 have been known as enzymes that can be induced by PAHs and ethanol, respectively, and their genetic polymorphisms have been reported to correlate with the metabolisms of various PAHs^{16, 21)}. In this study, the 8-OHdG level in leukocytes did not differ according to the genetic polymorphisms of *CYP1A1* and *CYP2E1*. Moreover, the 8-OHdG level in leukocytes correlated with urinary 1-OHP in every polymorphic type of *CYP1A1* and *CYP2E1*. Nevertheless, the correlation was stronger in subjects with the *CYP2E1* c2/c2 or c1/c2 form ($R=0.42$, $p\text{-value} < .01$) than in those with the c1/c1 form ($R=0.29$, $p\text{-value} < .05$). The correlation coefficient was also slightly larger in subjects with the *CYP1A1* Ile/Val or Val/Val type ($R=0.39$, $p\text{-value} < .05$) than in those with the *CYP1A1* Ile/Ile type ($R=0.30$, $p\text{-}$

value < .05). In light of the fact that the *CYP2E1* c2/c2 genotype is associated with the high activity of CYP2E1 enzyme²⁸, and that aryl-hydrocarbon hydroxylase inducibility is higher in individuals with the *CYP1A1* Val/Val type than in those with the *CYP1A1* Ile/Ile type²⁹, these results suggest that ROS can be induced by the metabolism of PAHs by CYP2E1 or CYP1A1. Since the expression level of CYP2E1 is higher, but that of CYP1A1 is lower in Asians than in Caucasians³⁰, and CYP2E1 rather than CYP1A1 is the major phase I enzyme metabolizing PAHs in Koreans¹⁶, CYP2E1 would be more important than CYP1A1 in the 8-OHdG generation by PAHs in Koreans.

The GSTs are phase II enzymes involved in the protection of cells against oxidative stress by electrophilic metabolites³¹. In this study, the correlations between the 8-OHdG level, and urinary concentration of 1-OHP and 2-naphthol were significant in subjects with the *GSTM1* null-type, but not in those with the *GSTM1* positive-type. These results are in agreement with previous reports that the presence of PAHs-dGMP adducts in human lung tissue was associated with the *GSTM1* null-genotype^{32, 33}, and that the urinary 2-naphthol concentration was higher in *GSTM1* null subjects¹⁶. These facts lead to a suggestion that *GSTM1* would play a critical role in the mechanism protecting against oxidative stress induced by PAHs³¹.

The NAT2 enzyme has been known to detoxify carcinogenic aryl amines and PAHs³⁴, but some previous studies have suggested that rapid acetylators are more susceptible to colorectal cancer due to the activation of dietary heterocyclic amines^{35, 36}, and colorectal patients showed a higher frequency of the rapid acetylator genotype than controls³⁷. A greater risk of bladder cancer has also been reported in Korean rapid acetylators³⁸. In this study, rapid acetylators showed significant correlations between the 8-OHdG level in leukocytes and the urinary 1-OHP concentration. This suggests that N-acetylation of PAHs would increase ROS production.

Two missense-mutations on one allele of *UGT1A6* result in the T181A and R184S amino acid substitutions and in reduced activity against phenolics, such as 4-nitrophenol, 4-hydroxycoumarin and butylated hydroxyanisole²⁴. Nevertheless, the influence of the *UGT1A6* polymorphisms on the metabolism of PAHs and their clinical significance are not yet fully understood. In the present study, in individuals with the *UGT1A6* wild-type, the 8-OHdG level in leukocytes significantly correlated with the urinary 1-OHP concentration. These findings suggest the possibility that glucuronidation of PAHs by UGT1A6 converts the chemicals to metabolites, which can induce oxidative stress.

The level of the 8-OHdG was significantly higher in smoking rapid acetylators than in smoking slow or intermediate acetylators, and in individuals with the *UGT1A6* wild-type than in those with the *UGT1A6* mutant

type. There were significant correlations between the 8-OHdG level and urinary 1-OHP level in NAT2 rapid acetylators, subjects with the *UGT1A6* wild-type, and those with the *GSTM1* null type. The effective clearance of PAH metabolites by NAT2 or UGT1A6 in the bodies of NAT2 rapid acetylators and subjects with the *UGT1A6* wild-type would accelerate phase I reactions of PAHs which produce substrates for the NAT2 or UGT1A6 enzyme. If more ROS are formed by the phase I reactions which metabolize PAHs to the substrates for NAT2 or UGT1A6 than other phase I pathways, then the level of 8-OHdG could be increased in rapid acetylators and individuals with *UGT1A6* wild-type. These indirect effects of phase II enzymes should be considered in the interpretation of the differences in the 8-OHdG level and in correlations between the 8-OHdG level and urinary 1-OHP level according to the genotype of *NAT2* or *UGT1A6*.

In all subjects, and in particular those with any genotype of *CYP1A1* or *CYP2E1*, the genotype of a rapid acetylator, or the *UGT1A6* wild-type, the level of 8-OHdG in leukocytes correlated with urinary 1-OHP, but not with 2-naphthol. There is the possibility that more ROS is produced by high molecular weight PAHs than by small-sized ones. The fact that naphthalene is absorbed mainly through the respiratory tract leads to another possible explanation: that PAHs absorbed via the GI tract or the skin show signs of greater oxidative damage than those absorbed through the respiratory tract³⁹.

In conclusion, there is a significant correlation between the 8-OHdG level in leukocytes and the urinary 1-OHP concentration in the population not occupationally exposed to PAHs. This relationship is affected by genetic polymorphisms in PAH metabolic enzymes.

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